

Normalization Methods for qPCR

Real-time PCR has been used for gene expression analysis for over a decade (Heid et al. 1996, Higuchi et al. 1992). The advent of better reagents and better techniques for assay design has increased the accuracy and efficiency of the nucleic acid quantification process, making quantitative PCR (qPCR) an even more powerful tool for gene expression studies.

Most gene expression assays are based on the comparison of two or more samples and require uniform sampling conditions for this comparison to be valid. Unfortunately, many factors can contribute to variability in the analysis of samples, making the results difficult to reproduce between experiments.

Variability is most often related to events upstream of the qPCR assay itself — namely, the quantity and quality of the extracted sample and the reverse-transcription efficiency (Fleige and Pfaffl 2006). Not only can the quantity and quality of RNA extracted from multiple samples vary, but even replicates can vary dramatically due to factors such as sample degradation, extraction efficiency, and contamination (Perez-Novo et al. 2005). The reverse-transcription efficiency can vary due to sample concentration, RNA integrity, the reagents used, and the presence of contaminants. Because the amount of starting material may vary greatly from sample to sample, accurate analysis requires that the samples be normalized.

This article describes two methods of sample normalization for accurate comparison of genes of interest: normalizing to input RNA and normalizing to a reference gene that has little variability as a function of treatment or due to the normal life cycle of the organism.

Normalization to Input RNA

Normalization to input RNA implies starting with the same amount and quality of material in each sample. This is typically done by measuring the absorbance at 260 nm (A_{260}) (Sambrook et al. 1989). This method cannot determine the integrity of the RNA molecules, however, because whole RNA and degraded RNA absorb light equally. Therefore, a secondary analysis, typically on a formaldehyde agarose gel, is required to determine whether there is degradation. Microfluidic electrophoresis on the Experion™ system is faster and requires much less RNA for this assessment than traditional agarose gel electrophoresis.

To illustrate the use of normalization in qPCR, we monitored the expression of four genes: annexin A3, CAPZB, cofilin, and destrin, in HeLa cells subjected to different treatments. Two sets of samples were transfected with small interfering RNAs (siRNAs, Act8 and Act9) that targeted different regions of the β -actin gene. A third set was transfected with siRNA targeting Green Fluorescent Protein (GFP) as a nonspecific control that is absent in HeLa cells. Two additional control samples were treated with lipid transfection reagent or buffer only. All analyses were performed using standard curves to determine individual amplification efficiencies.

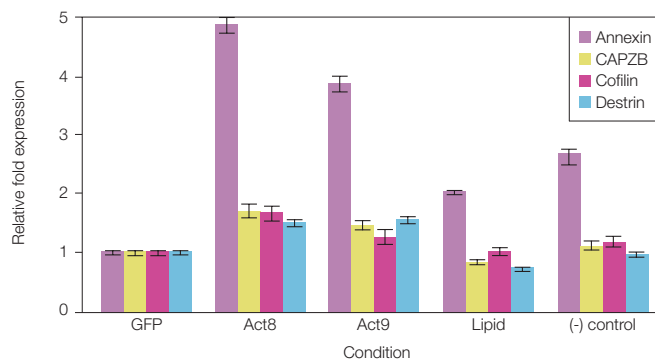


Fig. 1. Gene expression analysis of four genes across five conditions normalized to initial amount of starting RNA. GFP, Act8, Act9, treated with corresponding siRNA; Lipid, treated with lipid reagent; (-), control, treated with buffer only.

The relative expression of the four genes of interest across treatments was normalized to the amount of input RNA (Figure 1). This analysis showed that annexin A3 expression increased approximately 2- to 5-fold depending on the treatment. In addition, CAPZB, cofilin, and destrin displayed changes in expression varying from slightly below normal expression levels to a 50% increase in expression level.

Normalization to a Single Reference Gene

Although normalizing gene expression to the input amount of RNA ensures that equivalent amounts of RNA are compared, it cannot compensate for variations in the efficiency of reverse transcription, which is required to produce cDNA for PCR. Therefore, researchers often normalize expression levels of genes of interest to that of a reference gene. This can remove inaccuracies due to variations in reverse-transcription efficiency because the mRNA of the reference gene is reverse-transcribed along with that of the gene of interest.

Housekeeping genes such as β -actin, tubulin, GAPDH, and 18S ribosomal RNA have often been used as reference genes for normalization, with the assumption that the expression of these genes is constitutively high and that a given treatment will have little effect on the expression level.

To illustrate normalization to a reference gene, the data presented in Figure 1 were analyzed again, this time normalizing to the expression level of 18S RNA (Figure 2). The results of this analysis were qualitatively similar to those of the analysis using input RNA as the normalizer, but the calculated increases in expression were greater. For example, the expression level of annexin A3 in the Act8-treated sample showed a 4.9-fold increase when normalized to input RNA, but showed a 6.3-fold increase when normalized to 18S RNA expression. An obvious question is, which analysis is more accurate?

The question of which reference gene to use for normalization remains a key issue of debate. While it is agreed that the ideal reference gene is one that does not vary as a function of

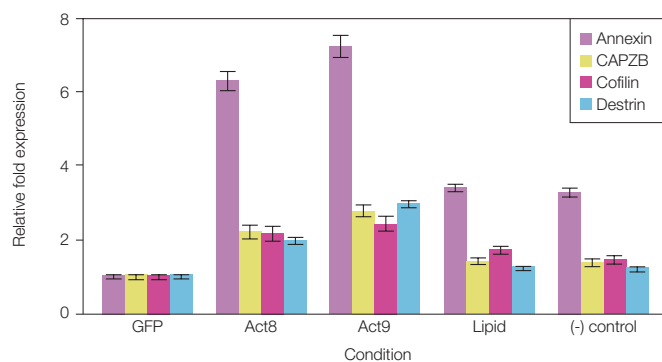


Fig. 2. Gene expression analysis of four genes across five conditions normalized to 18S ribosomal RNA.

treatment or condition, it is often difficult to identify even a single gene that meets this criterion (Glare et al. 2002, Kamphuis et al. 2005, Schmittgen and Zakrajsek 2000, Thellin et al. 1999). This difficulty is illustrated in Figure 3, which shows an analysis of three commonly used reference genes, 18S, GAPDH, and α -tubulin, from the same input. Although equivalent starting amounts of RNA were used, the expression levels of the three genes varied considerably depending on the treatment. These variations are unlikely to have resulted from errors in the starting amounts of RNA, because some of the genes are expressed at higher levels than the control, while others are expressed at lower levels. Therefore, these data indicate that genes that are generally considered to be housekeeping genes may in fact be expressed at variable levels across treatments or tissues.

Normalization to Multiple Reference Genes

A more accurate strategy for normalization has been proposed by Vandesompele and colleagues (2002). Instead of basing the normalization on a single reference gene that may or may not fluctuate, they propose carefully selecting a set of genes that display minimal variation across the treatment, determine the geometric mean of these genes, and normalize the gene(s) of interest to the geometric mean.

Figure 4 shows the expression levels of the four genes of interest across five treatments, as calculated using the multiple reference gene normalization strategy. This analysis reveals that treatment with either of the two siRNAs targeting β -actin (Act8 or Act9) increased annexin A3 expression levels to about 5.5-fold

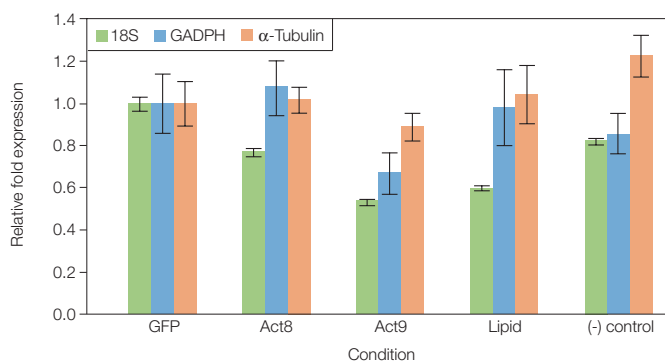


Fig. 3. Gene expression of three reference genes normalized to input amount of RNA.

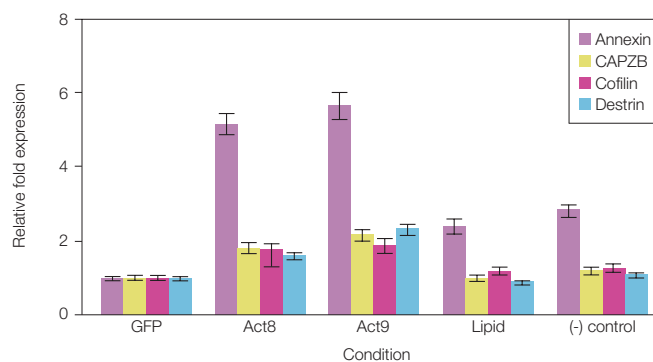


Fig. 4. Gene expression analysis of four genes across five conditions normalized to the geometric average of the three reference genes.

over basal level, and increased the expression of CAPZB, cofilin, and destrin to about 2-fold greater than basal levels. Annexin A3 also showed increased expression with lipid and buffer control treatments, but expression levels of the other three genes were similar in these controls to levels in the sample treated with nonspecific siRNA (GFP).

Summary

Proper normalization is essential for obtaining accurate gene expression studies. There are many strategies available for normalization, and with proper controls and replicates, all can be valid. The most comprehensive strategy uses a normalization factor calculated from the geometric mean of multiple reference genes.

To simplify data analysis, iQTM5 and MyiQTM real-time PCR detection systems come with analysis software that permits normalization to a standardized input amount, to a single reference gene, or to the geometric mean of multiple reference genes. Additionally, the software can take individual assay efficiencies into consideration, as well as combine multiple data sets to generate a complete gene study.

References

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